



ELSEVIER

JOURNAL OF
CHROMATOGRAPHY B

Journal of Chromatography B, 774 (2002) 205–214

www.elsevier.com/locate/chromb

Simultaneous quantification of prostaglandins, isoprostane and thromboxane in cell-cultured medium using gas chromatography–mass spectrometry

Hiroki Tsukamoto, Takanori Hishinuma*, Tsuyoshi Mikkaiichi, Hironori Nakamura,
Tohru Yamazaki¹, Yoshihisa Tomioka, Michinao Mizugaki

Department of Pharmaceutical Sciences, Tohoku University Hospital, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

Received 24 October 2001; received in revised form 28 March 2002; accepted 10 April 2002

Abstract

We have developed a simultaneous quantification method for prostaglandin (PG) E₂, PGD₂, PGF_{2α}, 8-epi-PGF_{2α}, 6-keto-PGF_{1α} and thromboxane (TX) B₂. Using [3,3,4,4-²H₄]PGE₂, [3,3,4,4-²H₄]PGD₂, [3,3,4,4-²H₄]8-epi-PGF_{2α}, [3,3,4,4-²H₄]PGF_{2α}, [3,3,4,4-²H₄]6-keto-PGF_{1α} and [18,18,19,19-²H₄]TXB₂ as internal standards (I.S.), the eicosanoids and their I.S. were simultaneously extracted by solid-phase extraction from cell-cultured medium, derivatized to methyl ester/methoxim/tert.-butyldimethylsilyl ether derivatives and analyzed using gas chromatography–mass spectrometry in the selected ion monitoring mode. The accuracy for the added eicosanoids ranged from 92 to 113%, and coefficients of variation ranged from 0.1 to 12.2%. Increased eicosanoids in RAW264.7 and U937 cells stimulated by lipopolysaccharide were suppressed by NS-398 and indometacin. This simultaneous quantification method can be applied routinely for assaying eicosanoids in vitro. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Prostaglandins; Isoprostane; Thromboxane

1. Introduction

Prostanoids, which include prostaglandins (PG) and thromboxane (TX), are produced from arachidonic acid by cyclooxygenase (COX) [1,2]. Isoprostanes such as 8-epi-PGF_{2α} are families of PG isomers [3,4]. 8-Epi-PGF_{2α} is produced in a free

radical catalyzed reaction from arachidonic acid and is COX-independent [3,4]. These eicosanoids have similar molecular structures, but have various biological effects [1,2]. For example, PGE₂ contributes to inflammation and carcinogenesis [1]. PGD₂ functions as a neurotransmodulator of several central actions such as the sleep–wake cycle [5], induces bronchoconstriction and acts as an allergic mediator [2]. TXA₂ and PGI₂, which have short half-lives, are automatically converted to TXB₂ [6,7] and 6-keto-PGF_{1α} [6], respectively. TXA₂ has aggregatory and vasoconstrictory effects [1,7]. In contrast, PGI₂ has anti-aggregatory and vasodilatory effects [6]. PGF_{2α} contributes to parturition and bronchoconstriction

*Corresponding author. Tel.: +81-22-717-7527; fax: +81-22-717-7545.

E-mail address: lahishi@mail.cc.tohoku.ac.jp (T. Hishinuma).

¹Present address: Department of Vascular Biology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan.

[1,2]. 8-Epi-PGF_{2 α} is formed in a free radical catalyzed reaction from arachidonic acid in phospholipids and is hydrolyzed by phospholipase A₂ [3,4]. It induces vasoconstriction in the pulmonary circulation, mitogenesis and platelet shape change [3]. These eicosanoids have pathological effects and the abnormalities they produce have been implicated in a wide range of diseases, including diabetic vascular complications [8], atherosclerosis [8], allergic asthma [2] and rheumatoid arthritis [9]. It is important to clarify the mechanism of eicosanoid production. Therefore, the establishment of a reliable microdetermination method is required to investigate the mechanism of eicosanoid production.

The quantification of eicosanoids is often performed using high-performance liquid chromatography-immunoassay techniques [10–16]. However, these techniques have some problems, including the use of a radioactive compound, a complicated procedure, low sensitivity and cross-reactivity. Recently, gas chromatographic-mass spectrometric methods were established and applied to biological samples [9,10,12–14,17–19]. However, these methods require complicated procedures. Furthermore, only one eicosanoid could be quantified at a time because different derivatization and purification procedures were required for each eicosanoid.

In this study, we established a simple and effective simultaneous quantification method for six eicosanoids using gas chromatography-mass spectrometry (GC-MS). We then measured eicosanoids in RAW264.7 and phorbol 12-myristate 13-acetate (PMA)-treated U937 cells stimulated with lipopolysaccharide (LPS) in the presence or absence of NS-398, a COX-2 selective inhibitor, or indometacin, a COX non-selective inhibitor. We suggest that 8-epi-PGF_{2 α} is produced in a COX-dependent manner in vitro.

2. Experimental

2.1. Materials

PGE₂, PGD₂, 8-epi-PGF_{2 α} , PGF_{2 α} , 6-keto-PGF_{1 α} , TXB₂, [3,3,4,4-²H₄]PGE₂, [3,3,4,4-²H₄]PGD₂, [3,3,4,4-²H₄]8-epi-PGF_{2 α} , [3,3,4,4-²H₄]PGF_{2 α} , [3,3,4,4-

²H₄]6-keto-PGF_{1 α} , indometacin and NS-398 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). [18,18,19,19-²H₄]TXB₂ was a gift from Ono Pharmaceutical (Osaka, Japan). LPS (from *E. coli* 0111:B4) was purchased from Wako (Osaka, Japan). *tert*-Butyldimethylsilyl (TBDMS) imidazole was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Bond Elut C₁₈ and Bond Elut Si cartridges were obtained from Varian (Harbor City, CA, USA). Diazomethane was prepared from *N*-methyl-*N*-nitroso-*p*-toluene-sulfonamide (Nakalai Tesque, Kyoto, Japan). Methoxyamine was purchased from Sigma (St. Louis, MO, USA). Other solvents and reagents were of analytical grade.

2.2. GC-MS analysis

An AutoSpec-Ultima mass spectrometer (Micromass, Manchester, UK) was interfaced to an HP6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with an all-glass Vandenbergh-type solventless injector and a HP-5 column (Quadrex, CT, USA), which was a 30 m×0.32 mm ID. (film thickness, 0.25 μ m) fused-silica capillary column cross-linked with 5% phenyl methyl silicone. The temperature of the column oven was increased from 200 to 240 °C at rate of 40 °C/min and then to 320 °C at a rate of 8 °C/min and maintained for 0.5 min. The carrier gas was helium. The temperatures of the injection port and the transfer line were maintained at 320 and 300 °C, respectively, and that of the ion source at 250 °C. The ionization energy and the trap current were 70 eV and 300 μ A, respectively. The accelerating voltage was 8 kV.

Full scan mass spectra were acquired by scanning from *m/z* 200 to 800 with a scan time of 1 s and a resolution of 1000.

Selected ion monitoring (SIM) was performed as follows: the monitoring ions for PGE₂, [3,3,4,4-²H₄]PGE₂, PGD₂ and [3,3,4,4-²H₄]PGD₂ derivatives were *m/z* 566.37, 570.39, 552.35 and 556.37, respectively, and these ions were monitored from 08:20 to 09:30 (function 1). The monitoring ions for 8-epi-PGF_{2 α} , [3,3,4,4-²H₄]8-epi-PGF_{2 α} , PGF_{2 α} and [3,3,4,4-²H₄]PGF_{2 α} derivatives were *m/z* 653.45, 657.47, 653.45 and 657.47, respectively, and these ions were monitored from 09:31 to 10:20 (function

2). The monitoring ions for 6-keto-PGF_{1 α} , [3,3,4,4-²H₄]6-keto-PGF_{1 α} , TXB₂ and [18,18,19,19-²H₄]TXB₂ derivatives were *m/z* 698.47, 702.49, 698.47 and 702.49, respectively, and these ions were monitored from 10:21 to 11:20 (function 3). The ions at *m/z* 554.97, 654.96 and 704.96 from perfluorokerosene (Jeol, Tokyo, Japan) were monitored as lock mass at functions 1, 2 and 3 to ensure stability. SIM was performed with a resolution of 4000.

2.3. Cell culture

RAW264.7 cells were purchased from the American Type Culture Collection. U937 cells were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). These cells were cultured in RPMI1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin G, 100 μ g/ml streptomycin, 60 μ g/ml kanamycin, 2 mM L-glutamine, 1 mM pyruvic acid and 0.2% glucose at 37 °C with air/CO₂ (95:5).

RAW264.7 cells ($5 \cdot 10^4$ cells) were cultured with 1 ml of medium in 24-well plates. After 24 h incubation, LPS (1 μ g/ml) was added to the medium in the presence or absence of indometacin (10 or 100 nM) or NS-398 (10 or 100 nM). After 12 h incubation, the complete volume of cell-cultured medium (1 ml) was harvested and stored at –80 °C until assayed.

U937 cells ($1 \cdot 10^5$ cells) were cultured with 1 ml of medium in 24-well plates. After 12 h incubation, U937 cells were treated with 100 nM PMA. After 48 h treatment, the medium containing PMA was removed and fresh medium (1 ml) containing LPS (1 μ g/ml) in the presence or absence of indometacin (100 nM or 1 μ M) or NS-398 (10 or 100 nM) was added. After 12 h incubation, the complete volume of cell-cultured medium (1 ml) were harvested and stored at –80 °C until assayed.

2.4. Sample purification and derivatization procedure

To the complete volume of cell-cultured medium (1 ml), tetra-deuterium analogues (2.5 ng each) were added as internal standards (I.S.). The sample was passed through a Bond Elut C₁₈ cartridge precon-

ditioned with methanol (4 ml), followed by distilled water (6 ml). The cartridge was washed with distilled water (4 ml) and hexane (4 ml). The eicosanoid fraction was eluted with ethyl acetate (4 ml) and evaporated. The residue was dissolved in methanol (100 μ l) and incubated with diazomethane (500 μ l) for 5 min to form the methyl ester (ME). After evaporation, the residue was incubated with 3% methoxyamine/pyridine (100 μ l) for 45 min at 65 °C to form methoxim (MO). After drying with N₂ gas, TBDMS imidazole (12 μ l) was added and incubated for 60 min at 70 °C to form the silyl ether derivative. The reaction mixture was passed through a Bond Elut Si cartridge preconditioned with hexane (4 ml) and eluted with hexane–ethyl acetate (95:5, v/v, 4 ml), followed by evaporation. The residue was dissolved in hexane (30 μ l) and 5 μ l was injected into the GC–MS system.

2.5. Statistical calculations

Student's *t*-test was applied to the data. Differences were considered significant at *P* < 0.05.

3. Results

3.1. Derivatization and purification

We obtained chemically stable derivatives, that is ME-MO-TBDMS ether derivatives for PGE₂, PGD₂, 6-keto-PGF_{1 α} and TXB₂ or ME-TBDMS ether derivatives for 8-epi-PGF_{2 α} and PGF_{2 α} , using diazomethane, methoxyamine and TBDMS imidazole. We used a Bond Elut C₁₈ cartridge to concentrate and clean up the analytes from the cell-cultured medium, and a Bond Elut Si cartridge to remove the excess derivatization reagents and to concentrate the analytes. The empirical formulae and molecular masses of the derivatized analytes were C₃₄H₆₅N₁O₅Si₂ (*M*_w 623.44) for PGE₂ and PGD₂, C₃₉H₇₈O₅Si₃ (*M*_w 710.52) for 8-epi-PGF_{2 α} and PGF_{2 α} , and C₄₀H₈₁N₁O₆Si₃ (*M*_w 755.54) for 6-keto-PGF_{1 α} and TXB₂. The structures of the derivatized analytes are shown in Fig. 1. TBDMS ether derivatives are superior to other silyl ether derivatives, such as dimethylisopropylsilyl ether and trimethylsilyl ether, for enhancing GC separation and improving the

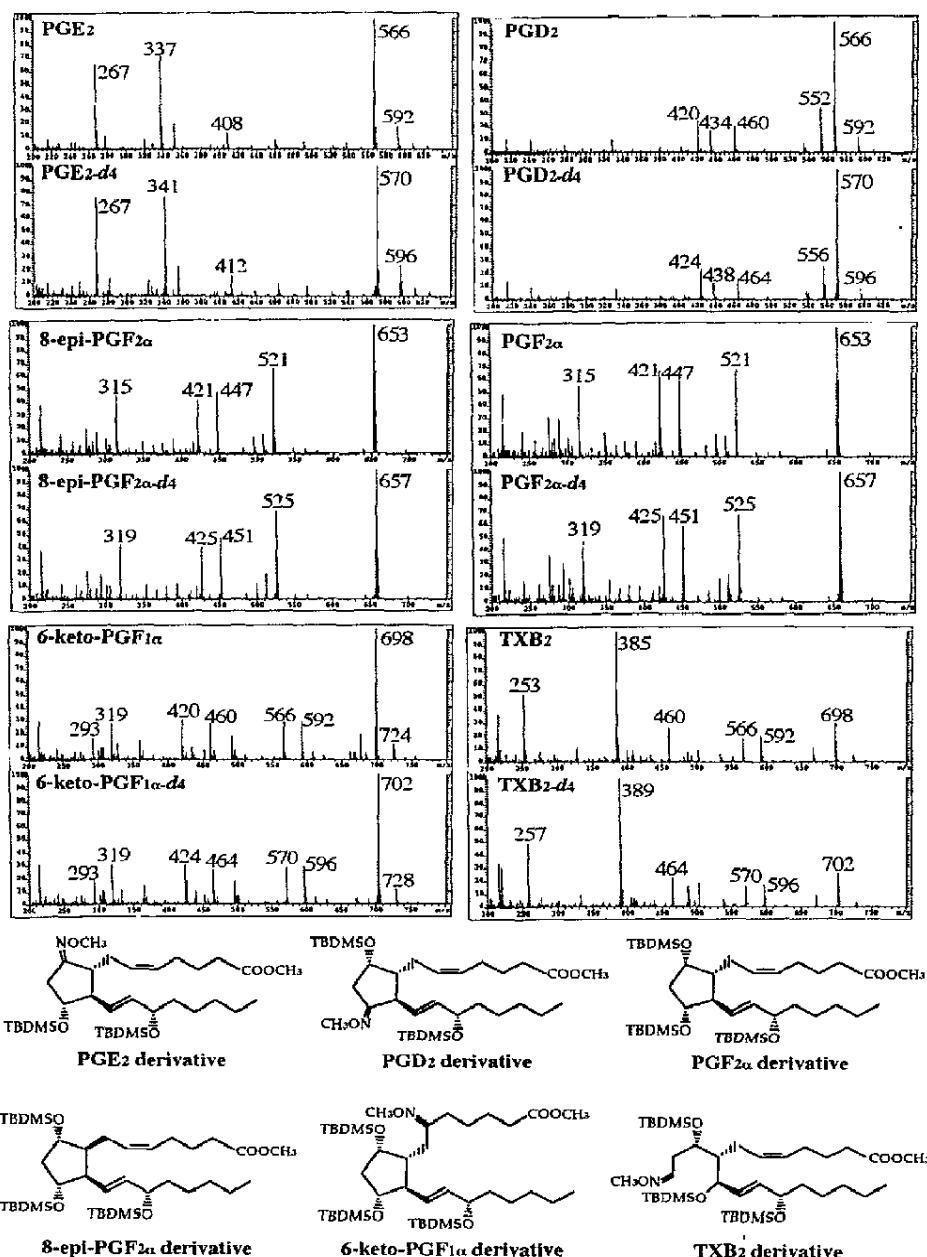


Fig. 1. Mass spectra and structures of PGE₂, PGD₂, 8-epi-PGF_{2α}, PGF_{2α}, 6-keto-PGF_{1α}, TXB₂ and their internal standard derivatives. Eicosanoids were derivatized to the ME-MO-TBDMS ether or ME-TBDMS ether derivative.

PM3006738924

Source: <https://www.industrydocuments.ucsf.edu/docs/phyx0001>

chemical stability of characteristic ions for quantification by GC–MS [20].

3.2. Mass spectra

Eicosanoids have similar molecular structures, so the derivatization procedure produced similar *m/z* fragment ions. Therefore, we had to choose the optimal fragment ions as monitoring ions for the analytes and separation on the GC column. The mass spectra of PGE₂, PGD₂, 8-epi-PGF_{2α}, PGF_{2α}, 6-keto-PGF_{1α}, TXB₂ and their I.S. are shown in Fig. 1. Each mass spectrum revealed a base peak at *m/z* 566, 566, 653, 653 and 698, corresponding to the [M–57]⁺ fragment ion ([M–C₄H₉]⁺) for PGE₂, PGD₂, 8-epi-PGF_{2α}, PGF_{2α} and 6-keto-PGF_{1α}, and at *m/z* 385, corresponding to the [M–370]⁺ fragment ion ([C_{12/20}]⁺) for TXB₂. We chose the [M–57]⁺ fragment ions ([M–C₄H₉]⁺), which were derived from the elimination of a *tert*-butyl radical, as monitoring ions for PGE₂ (*m/z* 566.37), 8-epi-PGF_{2α} (*m/z* 653.45), PGF_{2α} (*m/z* 653.45), 6-keto-PGF_{1α} (*m/z* 698.47) and TXB₂ (*m/z* 698.47). We chose the [M–71]⁺ fragment ion ([M–C₅H₁₁]⁺), which was derived from the elimination of a pentyl radical, as the monitoring ion for PGD₂ (*m/z* 552.35) instead of the [M–57]⁺ fragment ion ([M–C₄H₉]⁺). This appeared to be useful for the specific detection of PGD₂, because the [M–71]⁺ fragment ion was not observed in the mass spectrum of PGE₂.

3.3. Internal standards

We chose tetra-deuterium eicosanoid analogues as the I.S. for the analytes to establish a reliable microdetermination method. The advantages of using tetra-deuterium analogues as I.S. arise primarily from their identical chemical behavior to the native compounds, such as reaction with derivatization reagents, mass fragmentation pattern and chromatographic behavior. The mass spectra of [3,3,4,4-²H₄]PGE₂, [3,3,4,4-²H₄]PGD₂, [3,3,4,4-²H₄]8-epi-PGF_{2α}, [3,3,4,4-²H₄]PGF_{2α}, [3,3,4,4-²H₄]6-keto-PGF_{1α} and [18,18,19,19-²H₄]TXB₂ are shown in Fig. 1. The fragmentation patterns of the tetra-deuterium analogues are identical to those of the native compounds, except for the obvious mass unit shifts

caused by the four deuterium substitutions. Therefore, we chose the [M–57]⁺ fragment ions as monitoring ions for [3,3,4,4-²H₄]PGE₂ (*m/z* 570.39), [3,3,4,4-²H₄]8-epi-PGF_{2α} (*m/z* 657.47), [3,3,4,4-²H₄]PGF_{2α} (*m/z* 657.47), [3,3,4,4-²H₄]6-keto-PGF_{1α} (*m/z* 702.49) and [3,3,4,4-²H₄]TXB₂ (*m/z* 702.49), and the [M–71]⁺ fragment ion as the monitoring ion for [3,3,4,4-²H₄]PGD₂ (*m/z* 552.35).

3.4. Selected ion monitoring

We first optimized the temperature of the column oven to obtain a good separation of analytes with different retention times on the GC capillary column and with a short analysis time. We raised the temperature of the column oven from 200 to 240 °C at rate of 40 °C/min in the first step, from 240 to 320 °C at a rate of 8 °C/min in the second step and maintained this temperature for 0.5 min.

We tried to detect eicosanoids in cell-cultured medium using SIM mode at a resolution of 4000 to distinguish the four mass unit difference between the analytes and their I.S. Blank, zero control (=blank plus I.S.) and typical SIM chromatograms in cell-cultured medium are shown in Fig. 2. SIM chromatograms showed two separate peaks for PGE₂, PGD₂, 6-keto-PGF_{1α} and their I.S. and a single peak for 8-epi-PGF_{2α}, PGF_{2α}, TXB₂ and their I.S. with good signal-to-noise ratios. The peaks for the analytes were detected with the same retention times as that of their corresponding I.S. No serious interfering peaks were detected.

3.5. Calibration graphs

The calibration graphs for eicosanoids were generated from the GC–SIM of increasing amounts of authentic eicosanoids spiked with constant levels of tetra-deuterium analogues (2.5 ng each) as I.S. For PGE₂, PGD₂ and 6-keto-PGF_{1α}, we used the delayed larger peaks of double peaks. Linear calibration curves of PGE₂, 8-epi-PGF_{2α}, PGF_{2α}, 6-keto-PGF_{1α} and TXB₂ were obtained from 1 pg to 100 ng by plotting the peak area ratio of eicosanoids to I.S. versus their weight. A linear calibration curve was obtained for PGD₂ from 10 pg to 100 ng. The

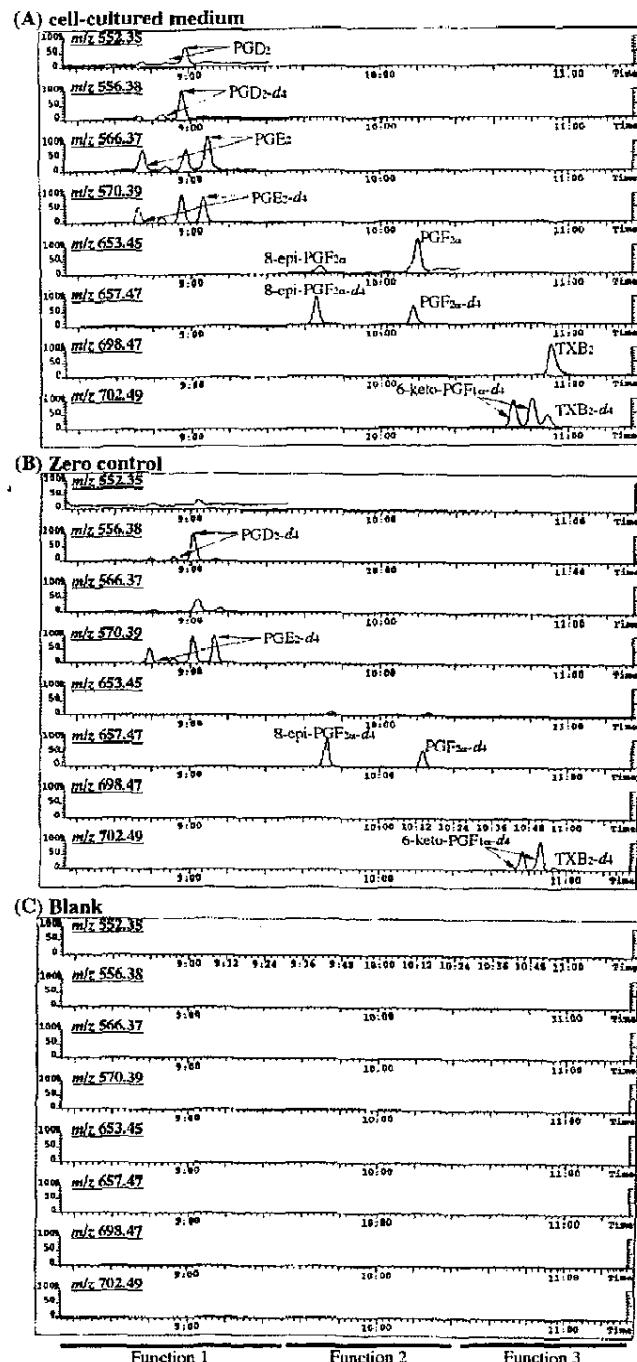


Fig. 2. SIM chromatograms of PGE₂, PGD₂, 8-epi-PGF_{2α}, PGF_{2α}, 6-keto-PGF_{1α}, TXB₂ and their internal standard derivatives in cell-cultured medium (A), zero control (B) and blank (C). SIM was performed by monitoring *m/z* 566.37 (PGE₂), 570.39 (PGE₂-d₄), 552.35 (PGD₂) and 556.38 (PGD₂-d₄) in function 1, *m/z* 653.46 (8-epi-PGF_{2α} and PGF_{2α}) and 657.47 (8-epi-PGF_{2α}-d₄ and PGF_{2α}-d₄) in function 2, and *m/z* 698.45 (6-keto-PGF_{1α} and TXB₂) and 702.49 (6-keto-PGF_{1α}-d₄ and TXB₂-d₄) in function 3.

PM3006738926

Table 1
Correlation coefficients and range of calibration curves

Eicosanoid	Correlation coefficient	Range
PGE ₂	0.998	1 pg to 100 ng
PGD ₂	0.994	10 pg to 100 ng
8-Epi-PGF _{2α}	0.999	1 pg to 100 ng
PGF _{2α}	0.999	1 pg to 100 ng
6-Keto-PGF _{1α}	0.993	1 pg to 100 ng
TXB ₂	0.997	1 pg to 100 ng

Tetra-deuterium analogues were used as internal standards.

correlation coefficients of the calibration curves ranged from 0.993 to 0.999 (Table 1).

3.6. Accuracy and precision

To examine the accuracy and precision of the present method, cell-cultured media (1 ml) spiked with 0, 500 and 5000 pg were prepared. These samples were analyzed in triplicate. The results are shown in Table 2. The accuracy for the added eicosanoids ranged from 92 to 113%. The coefficients of variation of samples spiked with 0, 500 and 5000 pg ranged from 0.1 to 12.2%. These findings

suggest that the present simultaneous quantification method is highly reliable with good accuracy and precision.

3.7. Application to cell-cultured media

We investigated the production of eicosanoids in RAW264.7 cells (Fig. 3) and PMA-treated U937 cells (Fig. 4) stimulated by LPS. RAW264.7 cells stimulated by LPS produced PGE₂, PGD₂, 8-epi-PGF_{2α} and PGF_{2α}. The major eicosanoid produced by RAW264.7 cells was PGD₂. These eicosanoids were significantly increased by LPS stimulation and were dose-dependently inhibited by 10 and 100 nM NS-398, a COX-2 selective inhibitor, and 10 and 100 nM indometacin, a COX non-selective inhibitor. PMA-treated U937 cells stimulated by LPS produced PGE₂, 8-epi-PGF_{2α}, PGF_{2α} and TXA₂. The major eicosanoid produced by PMA-treated U937 cells was TXA₂. These eicosanoids were significantly increased by LPS stimulation and were dose-dependently inhibited by 10 and 100 nM NS-398 and 100 nM and 1 μM indometacin.

Table 2
Accuracy and precision for the analysis of eicosanoid-spiked cell-cultured media

Sample (cell-cultured medium)	PGE ₂	PGD ₂	8-Epi-PGF _{2α}	PGF _{2α}	6-Keto-PGF _{1α}	TXB ₂
Non-spiked	1989	2068	90	301	3519	719
(pg/1 ml)	1980	2587	85	326	3509	750
	1972	2570	95	304	3513	703
Mean (pg/1 ml)	1980	2408	90	311	3514	724
SD (pg/1 ml)	9	295	5	13	5	24
CV. (%)	0.4	12.2	5.6	4.3	0.1	3.3
500 pg-spiked	2476	2938	521	731	4009	1271
(pg/1 ml)	2462	2875	585	887	4011	1297
	2517	3027	551	871	3969	1305
Mean (pg/1 ml)	2485	2947	552	830	3996	1291
SD (pg/1 ml)	29	76	32	86	24	18
CV. (%)	1.2	2.6	5.8	10.4	0.6	1.4
Accuracy (%)	101	108	92	104	96	113
5000 pg-spiked	6655	7235	4946	5251	8322	5961
(pg/1 ml)	6790	7357	5103	5615	8262	5467
	6439	7837	5094	5456	8151	6180
Mean (pg/1 ml)	6628	7476	5048	5441	8245	5870
SD (pg/1 ml)	177	318	88	183	87	365
CV. (%)	2.7	4.3	1.7	3.4	1.1	6.2
Accuracy (%)	93	101	99	103	95	103

PM3006738927

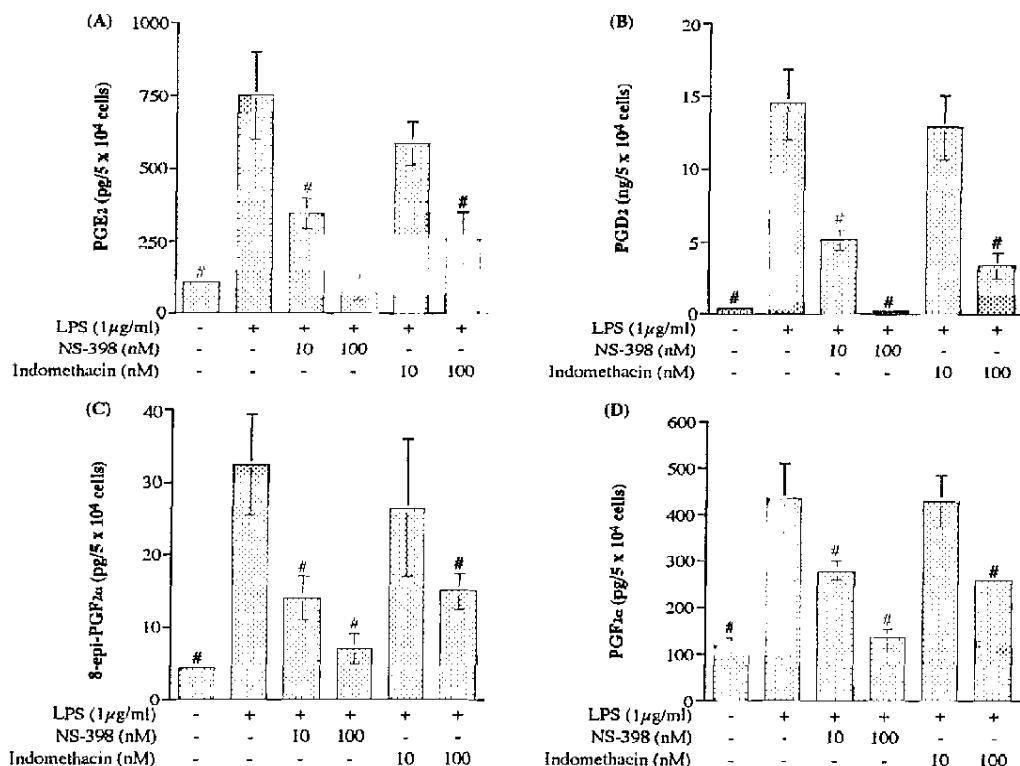


Fig. 3. Effects of NS-398 and indometacin on PGE₂ (A), PGD₂ (B), 8-epi-PGF_{2α} (C) and PGF_{2α} (D) production by RAW264.7 cells stimulated with LPS. RAW264.7 cells (5·10⁴ cells) were cultured with 1 ml medium for 24 h and stimulated with 1 µg/ml LPS in the presence or absence of indometacin (10 or 100 nM) or NS-398 (10 or 100 nM). After 12 h stimulation, the complete volume of the cell-cultured medium was harvested and assayed. Data are represented as pg or ng/initial cell number (5·10⁴ cells) as mean±SD of triplicate samples. Statistical significance is assessed versus LPS-treated RAW264.7 cells (#P<0.05).

4. Discussion

GC-MS is a powerful technique for the specific and quantitative measurement of extremely low levels of endogenous biological substance, since GC-MS combines separation of the analytes on a GC column and detection of ions by mass spectrometer. Because of its sensitivity and specificity, GC-MS is suitable for determining eicosanoids. In the GC-SIM mode, it is possible to choose and monitor specific fragment ions for each eicosanoid to determine the substrate more sensitively and specifically.

Microdetermination methods for eicosanoids using GC-MS have been reported previously [9,10,12–14,17–19]. However, with some of these previous

methods, only one eicosanoid can be quantified at a time; a few simultaneous quantification methods for eicosanoids have been reported [21–23]. Obata et al. [22] developed a simultaneous assay for PG and TX in cerebrospinal fluid using GC-MS. However, their method required a complicated purification procedure using four kinds of solid-phase columns. Obata et al. [22], Knott et al. [21] and Wübert et al. [23] did not report the accuracy and precision of their methods. Additionally, an I.S. for each analyte is not suitable [21,22]. With the present simultaneous quantification method, tetra-deuterium analogues were used as internal standards (I.S.) for each analyte and only two solid-phase columns were required for concentration and clean-up. In the SIM chromatogram, the analyte peaks were well separated with

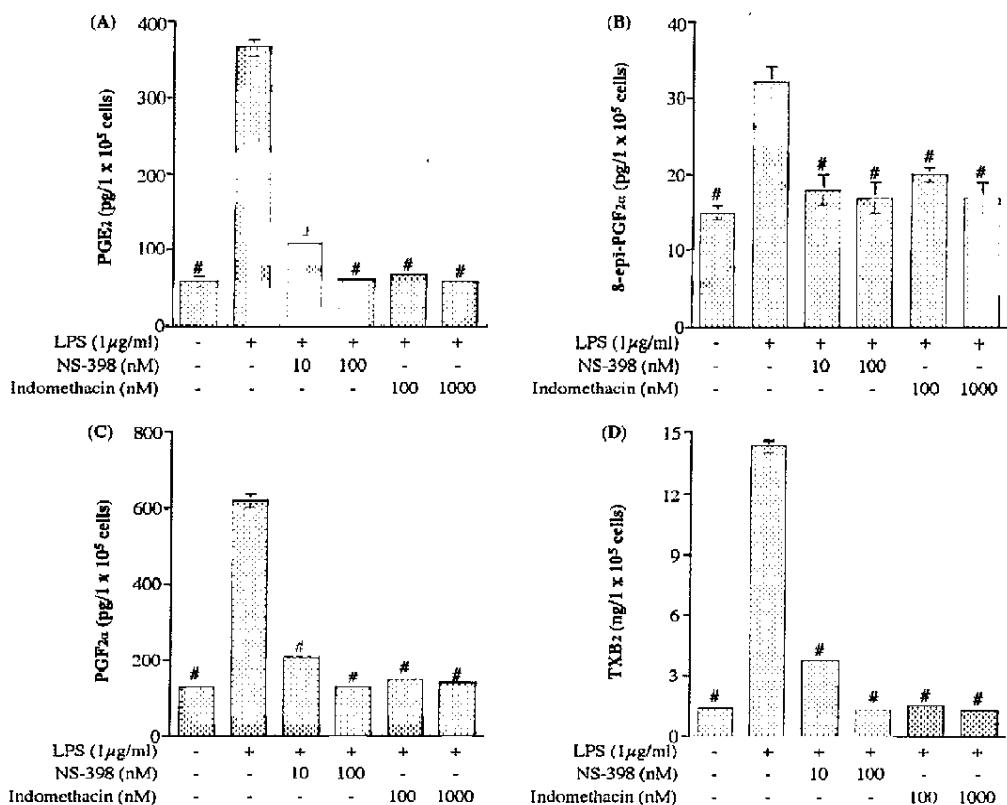


Fig. 4. Effects of NS-398 and indomethacin on PGE₂ (A), 8-epi-PGF_{2α} (B), PGF_{2α} (C) and TXB₂ (D) production by PMA-treated U937 cells stimulated with LPS. U937 cells ($1 \cdot 10^5$ cells) were cultured with 1 ml medium for 12 h and treated with 100 nM PMA. After 48 h treatment, medium containing PMA was replaced by PMA-free medium (1 ml). PMA-treated U937 cells were stimulated with 1 µg/ml LPS in the presence or absence of indomethacin (100 nM or 1 µM) or NS-398 (10 or 100 nM). After 12 h stimulation, the complete volume of cell-cultured medium was harvested and assayed. Data are presented as pg or ng/initial cell number ($1 \cdot 10^5$ cells) as mean \pm SD of triplicate samples. Statistical significance is assessed versus LPS-treated U937 cells. (# $P < 0.05$).

different retention times on the GC capillary column and no serious interfering peaks were detected. The analyte peaks were detected with the same retention times as that of their corresponding LS. We assumed that the two separate peaks for PGE₂, PGD₂, 6-keto-PGF_{1α} and their LS were derived from the *syn/anti*-isomers of methoxim derivatives. Good accuracy and precision was demonstrated. Therefore, the analyte peaks and their LS are selective and our simultaneous quantification method is more simple and reliable than their method.

Calibration curves ranging from 10 pg to 100 ng exhibited good linearity. This range covered the levels of eicosanoids produced by RAW264.7 ($5 \cdot 10^4$ cells) and U937 cells ($1 \cdot 10^5$ cells). We found that

increased 8-epi-PGF_{2α} production by stimulation of LPS in RAW264.7 and PMA-treated U937 cells was significantly inhibited by NS-398 and indomethacin. This finding suggests that 8-epi-PGF_{2α} was produced in a COX-dependent manner. It is considered that isoprostanes such as 8-epi-PGF_{2α} are formed COX-independently in a free radical catalyzed reaction from arachidonic acid esterified phospholipids in the cell membrane [3,4]. Therefore, 8-epi-PGF_{2α} is used as an index of lipid peroxidation [4]. Previously, Pratico et al. [3] also reported the COX-dependent production of 8-epi-PGF_{2α} and our findings support this. Considering the use of 8-epi-PGF_{2α} as an index of lipid peroxidation, it is necessary to clarify the pathway of 8-epi-PGF_{2α} production, that is, whether

8-epi-PGF_{2 α} is formed in a COX-dependent or -independent manner.

In conclusion, the present simultaneous quantification method using GC–MS is an efficient, specific, sensitive and accurate method for determining eicosanoids in cell-cultured media. Eicosanoids have been implicated in numerous pathophysiological conditions. The method described here is effective for assaying six eicosanoids from small numbers of cells and may be helpful in investigating the mechanism of eicosanoid production.

References

- [1] W.L. Smith, R. Langenbach, *J. Clin. Invest.* 107 (2001) 1491.
- [2] L. Pang, A. Pitt, D. Petkova, A.J. Knox, *Clin. Exp. Allergy* 28 (1998) 1050.
- [3] D. Pratico, J.A. Lawson, G.A. FitzGerald, *J. Biol. Chem.* 270 (1995) 9800.
- [4] J.D. Morrow, J.A. Awad, H.J. Boss, I.A. Blair, L.J. Roberts, *Proc. Natl. Acad. Sci. USA* 89 (1992) 10721.
- [5] Y. Urade, T. Tanaka, N. Eguchi, M. Kikuchi, H. Kimura, H. Toh, O. Hayaishi, *J. Biol. Chem.* 270 (1995) 1422.
- [6] T. Hishinuma, Y. Koseki, Y. Murai, J. Kotake, F. Ishii, K. Suzuki, M. Mizugaki, *Prostaglandins Other Lipid Mediat.* 55 (1998) 83.
- [7] S.S. Bhagwat, P.R. Hamann, W.C. Söll, S. Bunting, F.A. Fitzpatrick, *Nature* 315 (1985) 511.
- [8] M. Cocozza, T. Picano, U. Oliviero, N. Russo, V. Coto, M. Milani, *Stroke* 26 (1995) 597.
- [9] T. Hishinuma, H. Nakamura, T. Sawai, M. Uzuki, Y. Itabash, M. Mizugaki, *Prostaglandins Other Lipid Mediat.* 58 (1999) 179.
- [10] T. Tonai, K. Yokota, T. Yano, Y. Hayashi, S. Yamamoto, K. Yamashita, H. Miyazaki, *Biochim. Biophys. Acta* 836 (1985) 335.
- [11] D.K. Miller, S. Sadowski, D. DeSousa, A.L. Maycock, D.L. Lombardo, R.N. Young, E.C. Hayes, *J. Immunol. Methods* 81 (1985) 169.
- [12] J.Y. Westcott, S. Chang, M. Balazy, D.O. Stene, P. Pradelles, J. Maclouf, N.F. Voelkel, R.C. Murphy, *Prostaglandins* 32 (1986) 857.
- [13] S. O'Sullivan, M.J. Mueller, S.E. Dahlen, M. Kumlin, *Prostaglandins Other Lipid Mediat.* 57 (1999) 149.
- [14] Y. Hayashi, F. Shono, S. Yamamoto, W. Takasaki, A. Nakagawa, K. Watanabe, K. Yamashita, H. Miyazaki, *Anal. Biochem.* 187 (1990) 151.
- [15] H.J. Kramer, J. Stevens, W. Seeger, *Anal. Biochem.* 214 (1993) 535.
- [16] S. Basu, *Prostaglandins Leukot. Essent. Fatty Acids* 58 (1998) 319.
- [17] M. Ishibashi, Y. Nakagawa, N. Harima, T. Hishinuma, M. Mizugaki, *Biol. Mass Spectrom.* 23 (1994) 612.
- [18] T. Obata, K. Tomaru, T. Nagakura, Y. Izumi, T. Kawamoto, *J. Chromatogr. B* 746 (2000) 11.
- [19] M. Mizugaki, T. Hishinuma, G.S. Yu, K. Ito, M. Nishikawa, Y. Ohyama, M. Ishibashi, Y. Nakagawa, N. Harima, *J. Chromatogr. B* 658 (1994) 11.
- [20] T. Ohie, X. Fu, M. Iga, M. Kimura, S. Yamaguchi, *J. Chromatogr. B* 746 (2000) 63.
- [21] I. Knott, M. Raes, M. Dieu, G. Lenoir, M. Burton, J. Remacle, *Anal. Biochem.* 210 (1993) 360.
- [22] T. Obata, T. Nagakura, H. Maeda, K. Yamashita, K. Mackawa, *J. Chromatogr. B* 731 (1999) 73.
- [23] J. Wübert, B. Reder, A. Kaser, P.C. Weber, R.L. Lorenz, *Anal. Chem.* 69 (1997) 2143.

PM3006738930